D - 16.9
C - 9.6
A? - 31.4
1 IN? 36.3

Registry of Standard Biological Parts

Help:Protocols/Linearized Plasmid Backbones

< Help:Protocols

iGEM HQ provides linearized plasmid backbones for use during 3A Assembly and for shipping parts (pSB1C3). The following are the recommended protocols for using the linearized backbones and making your own.

If you're having issues or questions regarding linearized plasmid backbones contact us at **hq (at) igem . org**. We can also send more by request. Alternatively, you can find all of our standard plasmid backbones (pSB1C3, pSB1K3, etc) paired with BBa_J04450 in the Distribution Kit.

This protocol was developed by Tom Knight. Samples of standard Registry plasmid backbones prepared using this method are sent out in the DNA Distribution kits.

Contents

Why Linearized Plasmid Backbones?

Short single stranded DNA fragments will not ligate to 4 bp overhangs. By creating a very short overhang on a PCR of a plasmid backbone, the remnant, when cut with EcoRI and PstI is sufficiently short that it will not anneal at ligation temperature, and will therefore not ligate. This allows us to build high quality construction plasmid backbone without purifying away the cut fragments remaining after PCR.

Using the Linearized Plasmid Backbones

The DNA Distribution should come with a set of linearized plasmid backbones: pSB1A3, pSB1C3, pSB1K3.m1, and pSB1T3. The linearized plasmid backbones (25ng/ul at 50ul) should be stored at 4C or lower. Prior to ligation the plasmid backbones need to be cut with EcoRI and PstI.

Digest

- Enzyme Master Mix for Plasmid Backbone (25ul total, for 5 rxns)
  - 5 ul NEB Buffer 2
  - ~~0.5 ul BSA~~
  - 0.5 ul EcoRI-HF (http://www.neb.com/nebecomm/products/productR3101.asp)
  - 0.5 ul PstI (http://www.neb.com/nebecomm/products/productR0140.asp)
  - ~~0.5 ul DpnI~~ (http://www.neb.com/nebecomm/products/productR0176.asp) (Used to digest any template DNA from production)
  - ~~18~~ ul dH20 19

OR product

for plasm NO BSA yes .5ul DpnI le5uL dH2O

- Digest Plasmid Backbone
  - Add 4 ul linearized plasmid backbone (25ng/ul for 100ng total)
  - Add 4 ul of Enzyme Master Mix
  - Digest 37C/30 min, heat kill 80C/20 min

Ligation

- Add 2ul of digested plasmid backbone (25 ng) — ~~Cut~~ tetracycline & kanamycin (4 each).
- Add equimolar amount of EcoRI-HF SpeI digested fragment (< 3 ul)
- ~~Add equimolar amount of XbaI PstI digested fragment (< 3 ul)~~
- Add 1 ul T4 DNA ligase buffer (http://www.neb.com/nebecomm/products/productm0202.asp). **Note:** Do not use quick ligase
- Add 0.5 ul T4 DNA ligase (http://www.neb.com/nebecomm/products/productm0202.asp)
- Add water to 10 ul
- Ligate 16C/30 min, heat kill 80C/20 min
- Transform with 1-2 ul of product

6 LN kanamycin
2 LN tetra.

A - 3
C - 6

D - 3
F - 2

Note: For linearized plasmid backbones provided by iGEM HQ, a plasmid backbone with an insert of BBa_J04450 was used as template. As a result any red colonies that appear during your ligation may be due to the template as a background. Digesting with Dpn1 before use should reduce this occurrence.

Making Linearized Plasmid Backbones

Bulk Production

The following is the protocol that we used to create the linearized plasmid backbones shipped with the Spring 2011 DNA Distribution. The protocol is in 96 well format, but may be scaled down to suit smaller batches.

2012 Plasmid Backbone Production

2011 Plasmid Backbone Production

7/5/16

Today, the goal is to amplify the genes that the midshipmen were unable to amplify. The primer sets for these four genes have been labeled B, E, G, H.

The first step toward amplification is setting up a PCR. For our purposes, we will use a 50μL reaction volume and a high fidelity polymerase.

For one reaction tube (50μL)

- 25μL Q5
- 0.25μL Forward Primer
- 0.25μL Reverse Primer
- 0.5μL DNA
- 24μL Nanopure H_2O

Make one large master mix tube capable of aliquoting 11 reaction mixtures. Do not add primers since this is what we are testing. Create 2 Negative control tubes that contain DNA but no primer

11x

- 275 μL Q5
- 5.5 μL DNA
- 264 μL H_2O

Tubes

1. 16S Pos Control
2. Bin 5 (Labrinzia) Pos Control
3. Bin 7 Positive Control
4. Primer Set B
5. 16S Pos Control
6. Bin 5 Pos Control
7. Bin 7 Pos Control
8. Primer Set E
9. Primer Set G
10. Primer Set H
11. Negative Control
12. Negative Control

PCR #1

Tubes 1-4 and 12 will go into one thermocycler with the following protocol:

1. 98°C 30 sec
2. 98°C 10 sec
3. 72°C 30 sec
4. 72°C 30 sec
5. Repeat 2-4 35x
6. 72°C 3 min
7. 10°C hold forever

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PCR #2

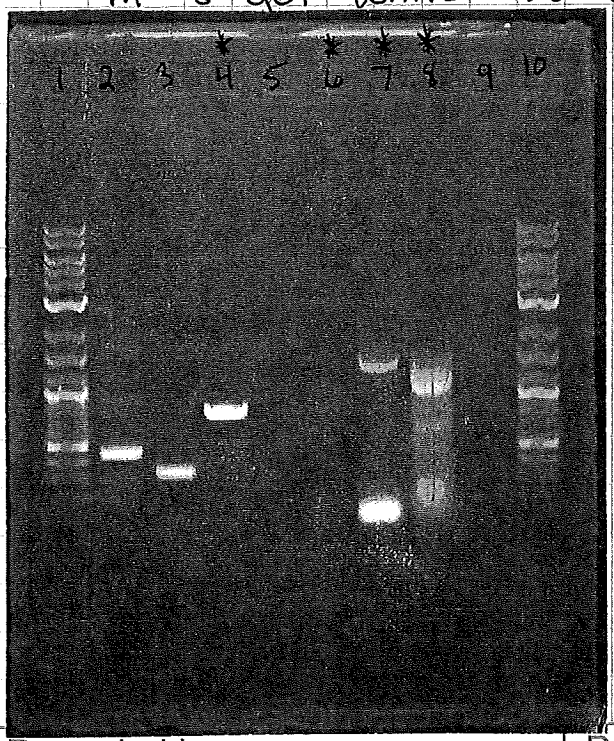
Tubes 5-11 will go into a different thermocycler with the following protocol:

1. 98°C 30 sec
2. 98°C 10 sec
3. 72°C 30 sec
4. 72°C 1 min 30 sec
5. Repeat 2-4 34x
6. 72°C 3 min
7. 10°C hold forever.

Note: After starting the thermocyclers, it was noticed that the extension time (step 4) for PCR #1 was not properly changed. Instead of being only 30 it was 1 min 30 sec.

Also, after thinking some more, the ~~extension~~ annealing temperature is not accurate for the positive contr

After running the PCR, 5μL was removed from each tube and r in a gel while the other 45μL ~~was~~ were used for PCR cle



Key

1.	Ladder		
2.	(2) Bin 5 Pos Control		~<500
3.	(3) Bin 7 Pos Control		
4.	(4) Primer Set B	*	771
5.	(5) 16S Pos Control		
6.	(8) Primer Set E	*	1415
7.	(9) Primer Set G	*	1405
8.	(10) Primer Set H	*	1485
9.	(11) Negative Control		
10.	Ladder		

To Page No

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7/6/16 After analyzing the gel, it was determined that lanes 4 and 7 contained the correct length band. However, lane 7 also contained a band that looked like primer dimers. ~~Both~~ ~~for~~ It was decided that only these two samples would go through PCR clean up.

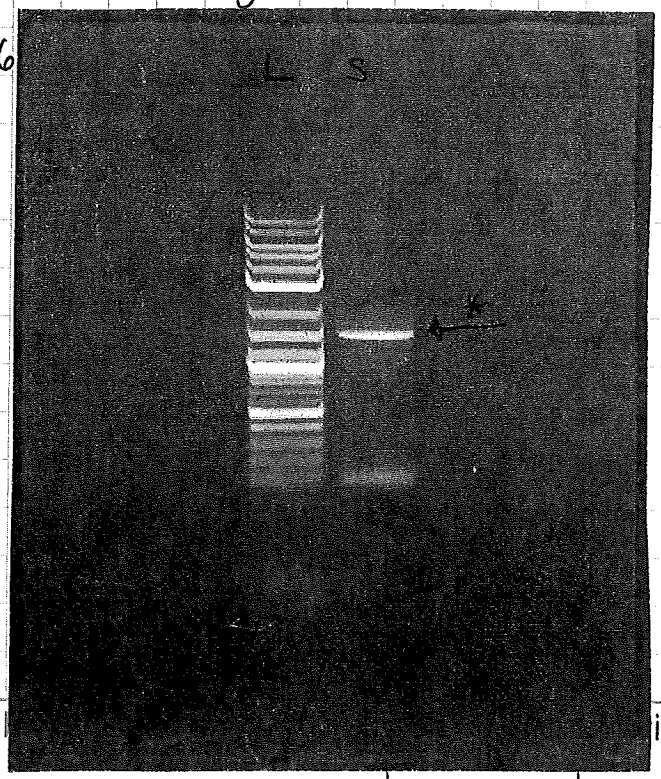
A PCR purification kit was used to isolate only the DNA and the samples were then analyzed in the Nanodrop.

Nano drop Results:

PCR tube #	DNA Conc.	260/280	260/230
4.	40 ng/μL	1.93	2.06
9.	35.8 ng/μL	1.85	1.92

The DNA for tube 4 has been properly prepared and is ready for additional steps.
The DNA for tube 9 is not ready for use. It will be run on a gel to see if the primer dimers have been eliminated. The proper band will be cut from the gel and a gel purification will be performed.

7/7/16



Using large wells and 50 μL of sample, w obtain the gel to th left.

L = Ladder
S = Sample

The starred band is the band interest which will be cut and purified.

Gel Purification:

mass of gel cut: 203 mg
Volume of QG = 3 x 203 = 609 µL
Volume of isopropanol = 203 µL

Use the Nanodrop to measure the concentration of DNA.

	Conc	260/280	260/230
PCR tube 9:	8.9 ng/µL	1:99	0.06

(8.9 ng/µL)(48 µL) = 427.2 ng Total

7/8/16 In order to complete transformations, the plasmid back bo must first be amplified through PCR. We will amplify pSB1K3.m1 and pSB1C3. In order to be able to accurately measure out the correct volume of DNA, the plasmid will be diluted into another tube.

$(25\ ng/\mu L)\ V_2 = (1\ ng/\mu L)(100\ \mu L)$

$V_2 = 4\ \mu L$

∴ Add 4 µL of concentrated plasmid to 96 µL of water and vortex!

7/11/16 PCR for plasmid amplification.

Final volume = 150 µL for each plasmid

Q5 = 75 µL
Primer F = 2.5 µL
Primer R = 2.5 µL
*Template = 3 µL
water = 67 µL
150 µL

* The template is either pSB1K3 or ~~pSB1K3~~ pSB1C3.

Aliquot 50 µL into PCR tubes. Should have a total of 6 tubes. 3 for C3 and 3 for K3

Recorded by: | Date | Verified by: | Date

Gel Purification:

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150 µL

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Aliquot 50µL into PCR tubes. Should have a total of 6 tubes. 3 for C3 and 3 for K3

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11/16

PCR protocol

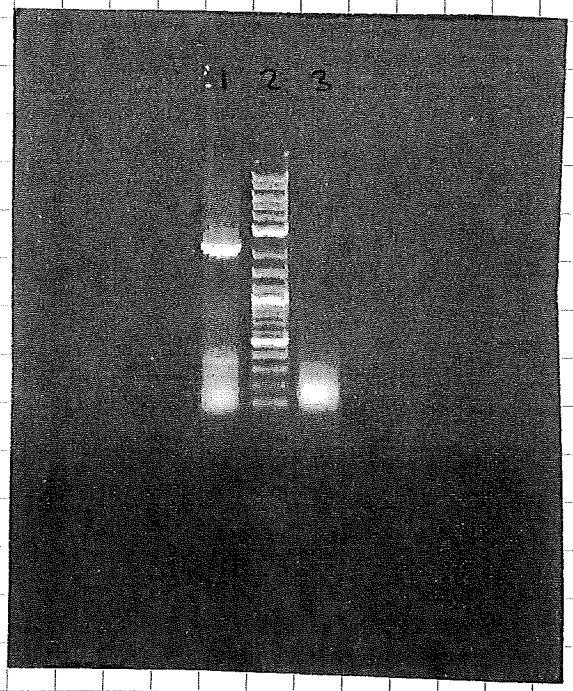
98°C for 30 sec
→ 98°C for 10 sec
* 66°C for 30 sec
72°C for 1 min 15 sec *
Repeat 35 x
72°C for 2 min
10°C for FOREVER

* Using the Q5 2x MM Tm calculator, the annealing temperature will be 66°C

* The length of K3 is 2204 bp. The length of C3 is 2070 bp. We will use 1 min 15 sec as the extension time.

Gel

1 pSB1K3.m1
2 Ladder
3 pSB1C3



It appears that pSB1K3.m1 did amplify. However, it is unknown why psB1C3 did not amplify. Another PCR should be run at a later date to check this.

A PCR clean up of pSB1K3.m1 was completed and the plasmid was put in the freezer over night.

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2/16

Nanodrop was used to quantify the amount of plasmid necessary for the digest with restriction enzymes.

Nano drop results for pSB1K3.m1

Conc: 283.8 ng/μL Total Volume = 48 μL
260/280: 1.90
260/230: 2.29

Calculation of how much volume to add to the digest. We want 1 μg of DNA.

Plasmid pSB1K3.m1:

$\frac{283.8\ ng}{\mu L}$ (V) = 1000 ng

V = 3.5 μL of plasmid.

Recipes For Digest (50 μL Reaction)

Plasmid
- 3.5 μL DNA
- 5 μL Buffer (cut smart)
- 1 μL Restriction Enzyme 1
- 1 μL Restriction Enzyme 2
- 40 μL Nanopure H_2O

Gene "B" *
- 25 μL DNA
- 5 μL Buffer (cut smart)
- 1 μL Restriction Enzyme 1
- 1 μL Restriction Enzyme 2
- 18 μL Nanopure H_2O

* conc = 40 ng/μL

$\frac{1000ng}{40\ ng/\mu L}$ = V

V = 25 μL

Gene "G" *
- 48 μL DNA
- 5 μL Buffer (cut smart)
- 1 μL Restriction Enzyme 1
- 1 μL Restriction Enzyme 2
- 0 μL H_2O

* conc = 8.9 ng/μL

$\frac{1000\ ng}{8.9\ ng/\mu L}$ = V

V = 112.4 μL

NOTE: since we only have 48 μL, we will use the whole volume for DNA and the reaction will be slightly over 50 μL.

To Page No.

7/12/16 To know which restriction enzymes to use for the double digest, we first looked at the primers made for the plasmid to see what restriction sites were present.

5'- ATGAATTCCAGAAATCATCACTTAGCG - 3'
(EcoRI)

5'- GCCGCTGCAGTCCGGCAAAAAA - 3'
(PstI)

Then, we looked to see if these sites were present in the primers for "B" and "G"

"B" primers

GAATTCGCGGCCGCTTCTAGATGAGCATCATGAAAGACAAGC
(EcoRI)

TACTAGTAGCGGCCGCTGCAGTCATGTTATTTCCACGTCCACG
(PstI)

"G" primers

GAATTCGCGGCCGCTTCTAGATGAGCGATCACACCATTACCAGC
(EcoRI)

TACTAGTAGCGGCCGCTGCAGTTATACGTCCCGGCTGCTGGCT
(PstI)

For primers "B" and "G", it may be difficult to get a complete digestion because the Eco RI site is at the end of the primer.
For the digest on the previous page (33), Restriction Enzyme 1 should be EcoRI and Restriction Enzyme 2 should be pst I

(p.33)
After completing the recipe for each tube, the tubes should be placed in an incubator at 37°C for one hour.

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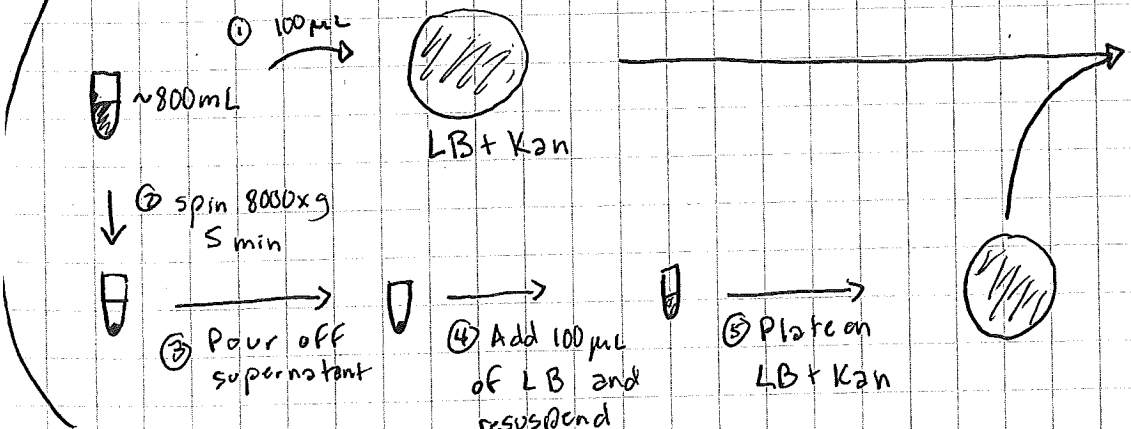
12/16 At the end of the incubation period, A PCR clean up should be performed to get rid of all of the tiny fragments of cut DNA. Then a nanodrop experiment should be run to quantify the DNA

Nanodrop Results for cut DNA:

	Conc	260/280	260/230	
"B"	9.3 ng/μL	2.52	3.67	
"K"	10.8 ng/μL	2.15	3.07	
"G" 1	1.6 ng/μL	2.80	-1.50	* Redo nano drop.
"G" 2	0.6 ng/μL	-0.87	-0.38	← (Trial 2)

1/13/16 Transformation Protocol

1. Remove competent cells from -80°C Freezer
2. Thaw on ice (20-30 min)
3. Add 10pg - 100ng (1-5 μL) of DNA to 50-100 μL of competent cells
4. Incubate on ice for 30 minutes
5. Heat shock at 42°C for 3 minutes
6. Cool on ice for 5 minutes
7. Add 750 μL of LB
8. Incubate and shake for 1 hour
9. Warm selection plates
10. Spread 100 μL onto 1 plate and spin down the rest
11. Resuspend pellet in 100 μL and plate
12. Incubate plates at 37°C overnight.



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7/19/16 The procedure on the previous page was completed for the tube labeled ligate K-B and the tube labeled ligate K-G.

Ligate K-B is a ligation of the plasmid K (pSB1K3.ml) and the insert B

Ligate K-G is a ligation of the plasmid K and the insert G

The ligation protocol was performed (7/13/16) before the transformation protocol. (I forgot to write the recipe in first). Add the following into a tube:

Ligate K-B	Ligate K-G
2 μL T4 buffer	2μL Buffer
2.3 μL plasmid (cut)	2.3μL plasmid (cut)
3.8 μL B (cut)	15μL G (cut)
10.9 μL H_2O	1μL Ligase
1 μL ligase	

The volumes of plasmid and insert were found using the ligation calculator on the NEB website.

Amount of plasmid needed for 20 μL Ligation is 25 ng

$(10.8\ ng/\mu L)\ V_K = 25\ ng$

$V_K = 2.3\ \mu L$

Amount of B (cut) needed for 20 μL Ligation is 26-44 ng. We decided to use 35 ng

$(9.3\ ng/\mu L)\ V_B = 35\ ng$

$V_B = 3.76\ \mu L$

Amount of G (cut) needed for 20μL Ligation is 48-80 ng (55 ng)

$(1.6\ ng/\mu L)\ V_G = 55\ ng$

$V_G = 34.38\ \mu L$

This value is not possible for a 20μL reaction

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7/13/16 Because we cannot use the required 34.38 µL of G, we will use the maximum volume that the 20 µL reaction will allow which is 15 µL.

After adding all of the required solutions, allow the tubes to incubate at room temperature for about an hour.

Then perform the transformation protocol on page 35.

7/14/16 After allowing the plates to incubate overnight, the plates were looked at to see if there was successful transformation and growth.

Unfortunately, there did not appear to be any growth on the plate with Ligate K-G (Transformed). There appeared to be 1 or 2 colonies on the plate with Ligate K-B (Transformed).

It may be that the competent cells are not so competent, so we will check to see if we can get the competent cells to work.

7/15/16 Key for what primers + DNA parts are what. (so we do not have to keep checking other papers)

In June, Mitchell and Maggie looked up which proteins in the MCL community are responsible for sensing + responding to voltage changes around the membrane. They then created primers in order to amplify the genes responsible for these proteins. These primer are what we have been testing.

7/15/16

Primer Set A

	Seq Name	Seq 5' to 3'
①	44000022-F	GAATTCGCGGCCGCTTCTAGATGAGCGATCCGAGCCCGCA
②	44000022-R	TACTAGTAGCGGCCGCTGCAGCATTGGGCCGTGTCTCTCACT

This primer set is for ArcA and the length of the DNA sequence is 770 bp

According to Mitchel & Maggie, this has been ligated, we will test by transforming using competent cells.

Primer Set B

③	70300038-F	GAATTCGCGGCCGCTTCTAGATGAGCATCATGAAAGACA
④	70300038-R	TACTAGTAGCGGCCGCTGCAGTCATGTTATTCCACGTCCACG

This primer set is for ArcA and the length of the DNA sequence is 771 bp

The midshipmen were unable to amplify this, so this is what I have been working on amplifying and expressing.

Primer Set C

⑤	69300040-F	GAATTCGCGGCCGCTTCTAGATGCCCAATCAATGCCCGGCGT
⑥	69300040-R	TACTAGTAGCGGCCGCTGCAGTTATAAGTCGATATCGAGTTTG

This primer set is for NIF and the length is 1623 bp. The ligated product will be transformed and tested.

15/16

Primer Set D

⑦ 657000001-F GAATTCGCGGCCGCTTCTAGATGAGCGGAACGGAAA CGGGCAG

⑧ 657000001-R TACTAGTAGCGGCCGCTGCAGTTACTTCGGCGCGCG TTTCGCA

This primer set is for PrrA and the length is 611 bp. The ligated product will be transformed and tested

Primer Set E

⑪ 678000023K-F GAATTCGCGGCCGCTTCTAGATGGCGCTGTTGAACAACC GCCA

⑫ 678000023K-R TACTAGTAGCGGCCGCTGCAGTCATGCGCGGACCGCGC

I do not know what this primer set is for, but the length of the DNA section is 1415 bp. This has been tested by PCR a few times with no result. It may be that this organism is not present in our mixed community. Therefore, we are not troubleshooting this at this time.

Primer Set F

⑬ 703000038K-F GAATTCGCGGCCGCTTCTAGATGATCAGCTGGTGGAAACGCAGT

⑭ 703000038K-R TACTAGTAGCGGCCGCTGCAGGTACTGACAAGGTACGGTCAAAAGG

this primer set is for ArcB & the length of the segment is 1446 bp. The ligated product will be transformed and tested.

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5/16

Primer Set G

(15) 56100000 4K-F GAATTCGCGGCCGCTTCTAGATGAGCGATCACACCATTACCAGC

(16) 56100000 4K-R TACTAGTAGCGGACCGCTGCAGTTATACGTCCCGGCTGCTGGCT

This primer set is for prrA which has a length of 1408 bp. The midshipmen were unable to amplify this, so I have been working on amplifying and expressing this.

Primer Set H

(17) 44000022K-F GAATTCGCGGCCGCTTCTAGATGGACGACACGTCCGACGC

(18) 44000022K-R TACTAGTAGCGGCCGCTGCAGCTATTTGCCCGCCCCGAGC

This primer set is for arcB which is 1489 bp. The first run with this primer set showed streaky bands, so I am trouble shooting from there.

8/16 Today, I would like to redo the PCR for pSB1C3 using a higher template to see if it will amplify.

Using 3 tubes for the plasmid ~~and 1 Negative Control Tube~~

Q5 = 75μL
Primer F = 2.5μL
Primer R = 2.5μL
Plasmid = 6μL
H_2O = 64μL
150μL *

Neg control
Q5 = 25μL
Primer F = 0.25μL
Primer R = 0.25μL
H_2O = 24.5μL
50μL

* Aliquot 50 μL into three different tubes and place all 3 tubes in the thermocycler

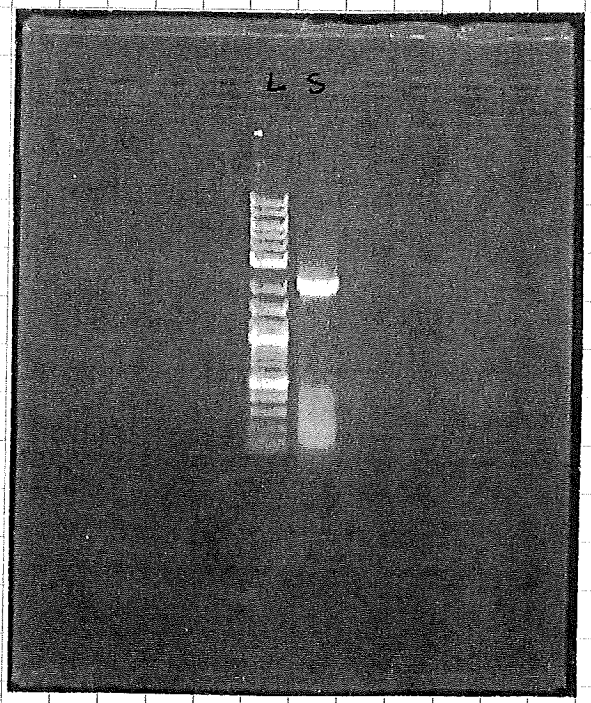
ded by: | Date | Verified by: | Date

1/18/16

PCR Protocol

98°C for 30sec
98°C for 10sec
66°C for 30sec
72°C for 1min 15sec
Repeat 35X
72°C for 2 min
10°C for FOREVER

After PCR is complete, run one tube of the product on a gel for approximately 40 minutes at 110 V



L = Ladder
S = pSB1C3

This result is good. It means that we got the plasmid amplified.

Now we will do a PCR cleanup and quantify using the nanodrop.

	Conc	260/280	260/230
C4	67.7	1.84	1.91
C5	366.7	1.39	0.85
C6	94.8	1.86	1.97

In addition, today, we are trying to retransform the two ligations into competent cells. Using the remaining ligation mixtures we performed the transformation protocol again for K-B and K-G. The plates were left to grow in the incubator overnight.

Also, Today we decided to take the one colony that was on the K-B plate, restreak it, and use part of it to run a colony PCR.

from 7/14/16

1. Using a pipet tip, take the colony off the plate
2. Make a small zig-zag on another plate
3. Swish the pipet tip into a PCR reaction mixture
4. Finish streaking the plate with a sterile stick. To Page No. ____

7/18/16 For the PCR mixture mentioned on the previous page, we are using a Green mix mixture

Recipe

- 12.5 µL Green Mix
- 12 µL Nuclease Free H_2O
- 0.25 µL F primer
- 0.25 µL R primer
- 1 colony from the tip of a pipet

Protocol

	95°C	3:00 minutes
35x	95°C	30 sec
	72°C	30 sec
	72°C	45 sec
	72°C	2 min
	10°C	∞

After PCR, run a gel to see if the colony contained the gene we were trying to clone in.
Look for pSB1K3.m1 :: arcA

It turns out that the gel was run for too long and the DNA ran off the end of the gel.

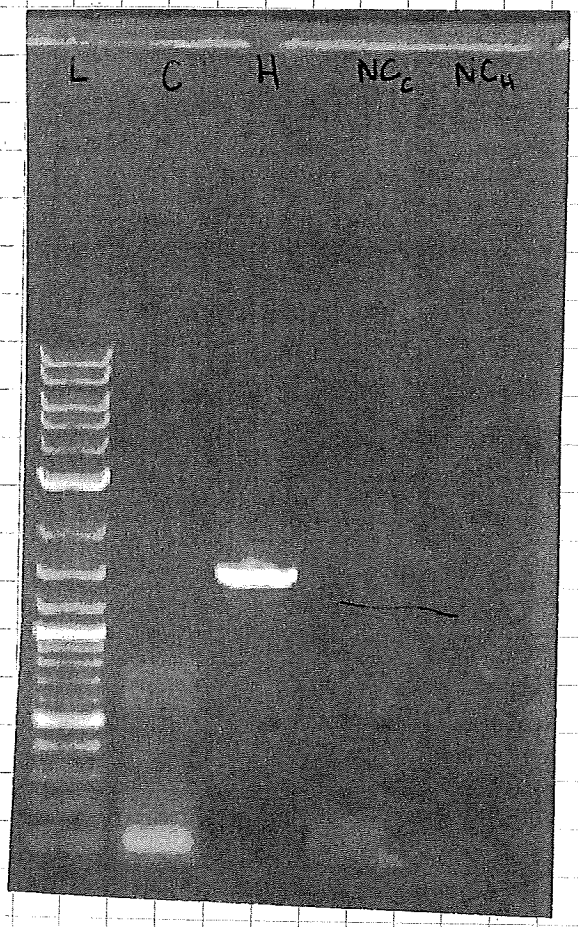
The last thing to do today is to try to amplify "H" again. We set up a PCR using Q5 and followed the exact same protocol as before (page 28)

This will be done on 7/19/16

Accomplished Today (summary)

- Gel from pSB1C3 showed the correct band
- One plate in the 37°C incubator overnight to screen for pSB1K3.m1 :: arcA
- Gel from plate (K-B) did not show anything because it ran off of the gel
- Four plates in the 37°C incubator to see if the transformations worked.

7/19/16 PCR for gene H and ran gel. It was discovered that the PCR from the colony had been left out overnight. We also ran this on the gel to see if it was still useful.



L Ladder
C Colony PCR (left out)
H gene H PCR product
NC$_C$ Neg Control Colony PCR (left out)
NC$_H$ Neg Control gene H

7/25/16 Four Midshipmen came today and started making plates. They should have notes for it.

7/26/16 Fitz and I redesigned the primers for arcA and arcB We have decided to just focus on these two ~~proteins~~ genes.

Goal: Promotor · Ribosome Binding Site · Gene · Reporter